Antisense basic fibroblast growth factor gene transfer reduces neointimal thickening after arterial injury

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Purpose: **To determine whether synthesis of endogenous basic fibroblast growth factor (bFGF) after arterial injury is critical to the intimal thickening response, intraluminal adenoviral gene transfer of an antisense bFGF (Ad.ASbFGF) transgene was used to inhibit the subsequent synthesis of bFGF protein after injury.**
Methods: **Sprague-Dawley rats underwent balloon catheter carotid artery injury and in vivo gene transfer. Isolated segments of rat common carotid artery were infected with an adenoviral vector encoding an antisense bFGF transcript at concentrations of 2×10^9, 1×10^{10}, or 1×10^{11} pfu/ml. Control rats were treated with either a control adenovirus encoding the β-galactosidase gene, (Ad.lacZ), at 1×10^{10}, or 1×10^{11} pfu/ml, or phosphate-buffered saline solution (vehicle). Two weeks after injury the rats were killed and perfusion-fixed. Cross-sectional areas of the carotid arterial intima and media were measured by planimetry, and the intima/media ratio (I/M) was calculated for each vessel.**
Results: **The mean I/M for each Ad.ASbFGF group and controls were compared and the significance assessed by analysis of variance. At two weeks after injury, the highest dose of Ad.ASbFGF, 1×10^{11} pfu/ml, resulted in a near total inhibition of thickening (I/M = 0.14 ± 0.04, mean ± SEM) when compared with phosphate-buffered saline solution alone (I/M = 0.99 ± 0.07), or Ad.lacZ 1×10^{10} pfu/ml (I/M = 1.01 ± 0.10) control treatments ($p < 0.01$). A tenfold lower dose of Ad.ASbFGF, 1×10^{10} pfu/ml, also caused significant reduction in intimal thickening (I/M = 0.39 ± 0.07, $p < 0.01$). Treatment with 2×10^9 pfu/ml Ad.ASbFGF did not significantly limit intimal thickening (I/M = 0.72 ± 0.12).**
Conclusions: **Inhibition of bFGF synthesis in vivo using an antisense RNA strategy significantly inhibits intimal thickening after arterial balloon injury. This study suggests that continued bFGF synthesis contributes to intimal thickening after arterial injury, and that antisense bFGF may represent an effective strategy in limiting restenosis after angioplasty. (J Vasc Surg 1997;25:320-5.)**

Neointimal hyperplasia is the most common form of vascular remodeling that occurs in response to various forms of vascular injury such as angioplasty of normal and atherosclerotic arteries.[1,2] The arterial wall responds to injury with smooth muscle cell (SMC) proliferation and migration of the vascular SMCs from the media to the intima, narrowing the diameter of the arterial lumen.[3-5] Restenosis after angioplasty is a frequent clinical problem that causes recurrent ischemia in both the peripheral and coronary circulations, and despite research in this area an effective therapy is lacking.[6,7]

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Both quiescent and proliferating arterial SMCs in vivo express basic fibroblast growth factor (bFGF). Olsen et al.[8] determined that immunohistochemical staining for bFGF is strongly positive in the uninjured artery, but falls significantly immediately after arterial injury. Western blot analysis of SMC extracts from injured and uninjured arteries detect a decrease in the total amount of bFGF in the injured arteries, thereby confirming the immunostaining results. During the 2 weeks after injury, the bFGF content gradually increases as predominately the 18kD isoform, rather than the 23kD isoform seen in uninjured SMCs.[8,9]

Lindner and Reidy[10] demonstrated that after arterial injury, addition of exogenous bFGF augments the reparative process of intimal hyperplasia. In contrast, they also determined that systemic injection of a single dose of neutralizing antibody against bFGF, administered at the time of arterial injury, decreases SMC proliferation by 80% at the time of death, 41 hours after injury (^{3}H thymidine uptake criteria). Unfortunately, the single dose of neutralizing antibody fails to reduce the intimal lesion that develops by 8 days. When neutralizing antibody is first given on the fourth and fifth days after arterial injury, it has no effect in reducing SMC ^{3}H thymidine uptake at 41 hours, nor intimal hyperplasia at 8 days.[10,11] These studies suggest that preformed bFGF released from fragmented SMCs at the time of injury may initiate SMC proliferation, but the role of endogenously synthesized bFGF at later times is unclear.

Fox and Shanley[12] found that when they infect cultured rat aortic SMCs with the adenovirally mediated antisense bFGF transgene (Ad.ASbFGF), the bFGF cellular content is significantly reduced on Western analysis. Control SMC cultures treated with adenovirally mediated β-galactosidase gene transfer (Ad.lacZ), or culture medium alone, maintain normal levels of bFGF. In addition, the SMC cultures infected with Ad.ASbFGF exhibit a dose-dependent reduction in survival compared with control cultures.[12]

It is not known whether after arterial injury, newly synthesized bFGF contributes to cellular events, leading to intimal hyperplasia in vivo. To study the role of bFGF synthesized in vivo after arterial injury, we exposed the carotid artery to a recombinant adenovirus encoding ASbFGF RNA, delivered locally as a single dose at the time of injury.

METHODS

Rat carotid artery model of intimal thickening and in vivo gene transfer

A total of 42 male Sprague-Dawley (SD) rats (300 to 350 gm; Harlan Sprague-Dawley Inc., Indianapolis, Ind.) were anesthetized with intramuscular injection of ketamine (50 mg/kg; Aveco Co., Fort Dodge, Iowa), xylazine (5 mg/kg; Mobay Corp., Shawnee, Kan.), and acepromazine (1 mg/kg; Aveco Co.). Routine common carotid balloon catheter arterial injury was performed as described by Clowes et al.[3] using a 2F Fogarty balloon catheter (Baxter Healthcare Corp., Santa Ana, Calif.).[13]

After arterial injury and phosphate-buffered saline solution (PBS) irrigation of the isolated arterial segment, a 50 μl volume of adenoviral vector in PBS, or PBS only, was injected into the lumen of the isolated artery and permitted to dwell for 20 minutes at 120 to 140 mm Hg pressure as described by Schulick et al.[13] Each treatment group consisted of eight rats receiving one of five treatments as follows: Ad.ASbFGF at 2×10^9 pfu/ml, Ad.ASbFGF at 1×10^{10} pfu/ml, Ad.ASbFGF at 1×10^{11} pfu/ml, PBS (vehicle alone) Ad.lacZ at 1×10^{10} pfu/ml, or Ad.lacZ at 1×10^{11} pfu/ml.

After treatment, the vector solution was withdrawn and the artery gently rinsed again with PBS, then blood flow was restored to the common and internal carotid arteries. The site of injury and treatment was marked proximally by a 7-0 nylon suture placed in adjacent muscle and delimited distally by the bifurcation of the common carotid artery. The neck incision was closed, and the animal was placed on a warming blanket until fully recovered from anesthesia. After recovery, the rats were fed normal chow and water ad libitum until they were killed. Animal care complied with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* (NIH publication no. 86-23, revised 1985).

Monitoring of instillation pressure

A pressure transducer coupled to a Sirecust 404-1 monitor (Siemens, Erlangen, Germany) was used to measure the delivery pressure of Ad.ASbFGF and control treatments. The treatment solution was injected at a pressure of 120 to 140 mm Hg as recommended by Schulick et al.[13]

Construction of antisense basic FGF adenoviral vector

The Ad.ASbFGF recombinant adenovirus was constructed by replacement of the β-galactosidase cDNA with a 1.1 kb rat bFGF cDNA (gift of Dr. Andrew Baird, Whittier Institute)[14] in the pAd.CMV lacZ shuttle vector (gift of Dr. James Wilson, University of Pennsylvania), followed by homologous recom-

bination[15] with the E1, E3 deleted, human adenovirus serotype 5 mutant dl7001[16] in human embryonal kidney 293 cells (American Type Culture Collection, Bethesda Md.). Recombinant virus was identified by polymerase chain reaction (PCR) amplification of recombinant plaques using one vector-specific and one insert-specific primer. The vector-specific primer (5′-AGA CAT GAT AAG ATA CAT-3′) corresponds to a region of the shuttle vector upstream of the insert cloning site but contains no adenoviral genomic sequence. The insert-specific primer (5′-GCT TCT TCC TGC GCA TCC-3′) corresponds to codons 37-42 of the rat bFGF coding sequence. The recombinant virus was twice plaque-purified and expression of the antisense transcript confirmed by reverse transcriptase polymerase chain reaction. Total cellular RNA (1 μg), isolated from SMCs infected with Ad.ASbFGF, was heat denatured and reverse transcribed using random hexamer primers (1 pmol) and 200 U reverse transcriptase (Boehringer Mannheim, Indianapolis, Ind.), according to the supplier's recommendations. Ten percent of the cDNA product was then amplified using 1 pmol each of a pair of bFGF-specific primers (5′ primer: 5′-GCA CAC ACT CCC TTG ATG GAC AC-3′; 3′ primer: 5′-GCT TCT TCC TGC GCA TCC-3′) that amplify a 125–base pair product. The PCR was performed using 1 U per reaction of Taq polymerase (Boehringer Mannheim) in a thermal cycler (Ericomp Inc., San Diego, Calif.) for 25 cycles using 1 minute steps. Denaturation was done at 94° C, annealing at 60° C, and extension at 72° C.

The recombinant adenoviral vector, Ad.CMV lacZ, encoding bacterial β-galactosidase (gift of Dr. James Wilson, University of Pennsylvania),[16] was used as a viral vector control in these experiments. The suspension vehicle, PBS (Bio-Whittaker, Walkersville, Md.), served as the second control.

Tissue preparation

Frozen tissue. All rats were killed with lethal doses of anesthesia. Two of eight rats from each treatment group were killed 4 days after injury and treatment. Tissue samples from those rats were quickly harvested, rinsed of blood, and frozen in OCT Compound (Miles Inc., Elkhart, Ind.) for testing the content of β galactosidase. The uninjured right carotid arteries served as baseline controls for gene transfer testing.

Formalin fixation. The remaining six of the eight rats in each treatment group were killed 2 weeks after injury, after which the vessels were perfusion fixed in situ at a perfusion pressure of 120 mm Hg. Normal saline solution was instilled first, followed by 10% formalin solution via a large-bore cannula placed retrograde in the abdominal aorta. The carotid vessels and aortic arch were harvested en bloc for further immersion fixation, paraffin embedding, hematoxylin-eosin staining, and subsequent morphometric analysis. All chemicals were of analytical grade quality. Cross-section samples of the injured carotid arteries were taken from the center of the injured and treated area.

Documentation of Ad.lacZ gene transfer to carotid tissue

Animals killed on day 4 after injury were evaluated for the presence of β-galactosidase activity, to indicate successful Ad.lacZ infection and β-galactosidase expression. Carotid artery specimens (including control and Ad.ASbFGF treated injured arteries) were processed with X-gal chromagen to detect the presence of β-galactosidase activity. Carotid artery tissue samples were thawed and rinsed with PBS to remove OCT. The tissues were immersed in PBS for 20 minutes, then fixed in 2.5% glutaraldehyde (Fisher Scientific, Fair Lawn, N.J.) for 15 minutes. Samples were rinsed twice in 1 mmol/L MgCl-PBS before incubation with the X-gal chromagen mixture (5-Bromo-4-chloro-3-Indolyl-β-D-galactopyranoside, Fisher Scientific, Malvern, Pa.) for 1 hour at 37° C. Tissues that stained positive for β-galactosidase appeared dark blue and contained blue cytoplasmic inclusions when magnified. Cross-section and enface preparations were examined at 10X and 15X with a Leitz dissecting photomicroscope (Leica Inc., Malvern, Pa.). Results were recorded as photomicrographs.

Morphometric analysis of arterial wall

Morphometric analysis was performed on cross-sectioned segments of carotid arteries. A light microscope (Leitz Laborlux-s, Leica Inc.), fitted with a computer interfaced image analyzer (Bioquant System IV, R&M Biometrics Inc., Nashville, Tenn., and Summa Sketch II Plus, Summagraphics Corp., Seymour, Conn.), was used to calculate the surface area of the arterial wall intima and media at a magnification of 125X. The intima to media ratio (I/M) was calculated on all perfusion-fixed carotid arteries harvested 2 weeks after injury and treatment. An I/M of 1.0 or greater implies that the intimal area is equal to or greater than the area of the media. An I/M of 0.15 indicates the intimal area is very small. The intimal cross sectional area of an uninjured rat artery approaches 0, as it is only one endothelial cell layer thick.

Table I. Comparison of mean intima to media ratios

Treatment (pfu/ml)	*n*	*mean I/M ratio ± SEM*	*p (vs lacZ 1 × 10¹⁰ pfu/ml)*	*p (vs PBS)*
PBS (control vehicle)	6	0.99 ± 0.07	NS	
Ad.lacZ (control 1 × 10^{10})	6	1.01 ± 0.10		NS
Ad.lacZ (control 1 × 10^{11})	6	1.08 ± 0.07	NS	NS
Ad.ASbFGF 2 × 10^{9}	6	0.72 ± 0.12	NS	NS
Ad.ASbFGF 1 × 10^{10}	6	0.39 ± 0.07	<0.01	<0.01
Ad.ASbFGF 1 × 10^{11}	6	0.14 ± 0.04	<0.01	<0.01

Comparison of mean I/M ratios by analysis of variance. *p* values calculated by the Tukey method of post hoc pairwise testing.

Statistical analysis

Two weeks after injury, the I/M was measured on all carotid arteries harvested from the formalin perfusion-fixed animals. The mean I/M was calculated for each group (± standard error of the mean) and were compared to one another by analysis of variance, followed by the Tukey method of post hoc pairwise testing. A p value of 0.05 or less was considered significant (Systat 5.01 for Windows, Systat Incorporated).

Toxicity analysis

Samples of liver, spleen, skeletal muscle, and aorta were harvested from the animals killed at 4 days after injury and treatment. Each treatment group, Ad.ASbFGF, Ad.lacZ, or PBS, contained two animals. The organs of animals treated with Ad.lacZ and PBS were processed with X-gal chromagen to identify uptake of the Ad.lacZ transgene by hematogenous dispersion. Tissues from all treatment groups were fixed in 10% formalin and embedded in paraffin. Routine histologic hematocylin and eosin sections of the organs were examined for microscopic pathologic changes.

RESULTS

Ad.lacZ transgene expression

Animals killed 4 days after injury and treatment were evaluated for the presence of β-galactosidase activity. Carotid arteries infected with Ad.lacZ at a concentration of 1 × 10^{10} pfu/ml, when processed with X-gal chromagen, demonstrated on cross-sectional view the depth of arterial wall penetration achieved by adenovirally mediated gene transfer. Expression of β-galactosidase was noted transmurally, with blue staining cytosolic inclusions in the SMCs of the media and adventitia. This finding demonstrates that the adenoviral vector, used to deliver the Ad.lacZ transgene, infects the SMCs of the arterial media when delivered with a pressure of 120 mm Hg after injury. No detectable endogenous β-galactosidase activity was noted in PBS or Ad.ASbFGF treated injured arteries after processing in X-gal chromagen.

Effects of Ad.ASbFGF in the rat carotid artery model

To investigate the effect of adenovirally mediated Ad.ASbFGF gene transfer on limiting the intimal thickening response to balloon injury, we instilled the recombinant vector into the lumen of the left carotid artery at the time of injury. At 2 weeks after injury and treatment, the I/M was measured after perfusion fixation. Significant inhibition of intimal thickening was observed in the groups treated with Ad.ASbFGF 1 × 10^{10} and 1 × 10^{11} pfu/ml when compared to PBS and Ad.lacZ controls (Table I). A dose-response effect was noted in the Ad.ASbFGF treated injured arteries. The lowest dose of AdASbFGF, 2 × 109 pfu/ml, resulted in a mean I/M of 0.72. The difference was not statistically significant from the PBS-treated arteries (I/M = 0.99), nor the Ad.lacZ treated arteries (I/M = 1.01). A moderate dose of Ad. ASbFGF, 1 × 10^{10} pfu/ml, resulted in a mean I/M of 0.39, and the highest dose of Ad. ASbFGF,1 × 10^{11} pfu/ml, had the greatest effect, with a mean I/M of 0.14. The degree of inhibition of intimal thickening with both of the higher doses of Ad.ASbFGF was statistically significant ($p < 0.01$). There was a trend toward a lower I/M in the group treated with the lowest dose of Ad.ASbFGF, though not statistically significant in this small sample size (n = 6). The six rats receiving treatment with Ad. lacZ virus at a concentration of 1 × 10^{11} pfu/ml showed no difference in the intimal thickening response (I/M = 1.00) when compared with rats treated with a lower dose of the same vector, Ad.lacZ 1 × 10^{10} pfu/ml (I/M = 1.01).

The photomicrographs in Fig. 1 demonstrate the inhibition of neointimal thickening in the injured artery by Ad.ASbFGF gene transfer compared with controls.

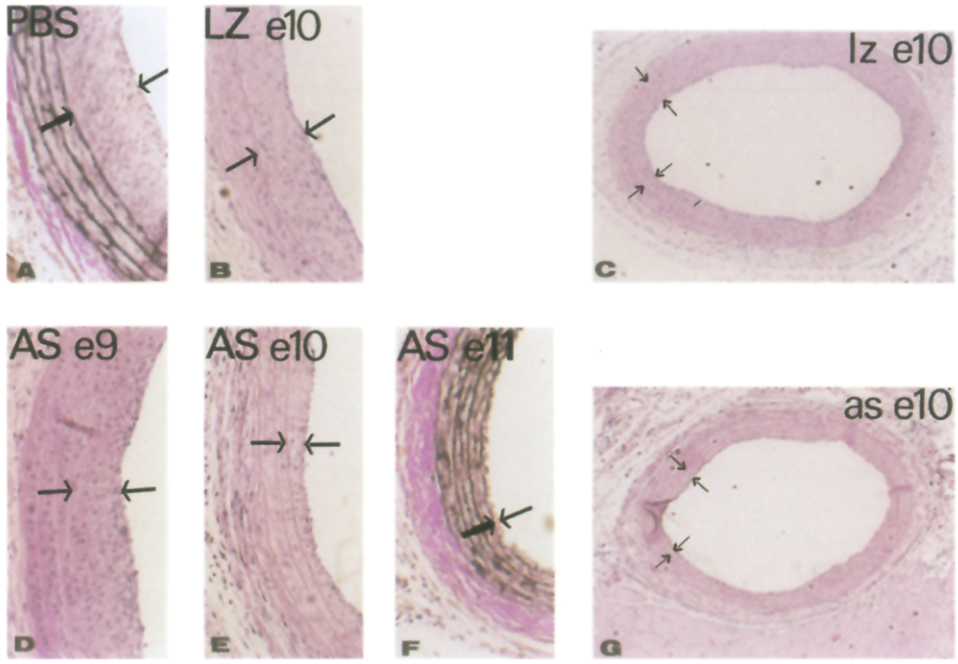

Fig. 1. Composite of photomicrographs of representative cross-sections from arteries treated with either control adenovirus transporting Ad.lacZ, the same adenovirus transporting Ad.ASbFGF, or vehicle alone (PBS). Control arteries are in top row (**A-C**); bottom row contains images of arteries treated with increasing doses of Ad.ASbFGF (left to right). In each micrograph, the neointima is demarcated between two *black arrows.* Treatment with the lowest dose of Ad.ASbFGF, 2×10^9 pfu/ml (**D**), does not significantly decrease neointimal thickening compared with control arteries *(top row).* Ad.ASbFGF at a slightly higher dose, 1×10^{10} pfu/ml (**E**), results in a reduction of the intimal thickening response by 66%. Highest dose of Ad.ASbFGF, 1×10^{11} pfu/ml (**F**), shows nearly negligible intimal thickening. The arteries depicted in images **C** and **G** were treated with the same concentration of adenovirus, 1×10^{10} pfu/ml, but **C** was treated with the Ad.lacZ gene, and **G** was treated with the Ad.ASbFGF gene. These images, taken at lower magnification, demonstrate that response to Ad.ASbFGF (a 66% reduction in neointimal hyperplasia) is uniform along lumen circumference (**G**). All tissues are stained with hematoxylin-eosin except for images **A** and **F**, which are prepared with elastin-collagen stain to demonstrate the elastin layers of the media. Images **A, B, D, E,** and **F** are magnified by 125X. Images **C** and **G** are magnified by 50X.

Toxicity of Ad.ASbFGF

Of the 42 rats included in this study, there were no postoperative deaths or complications. There was no clinical evidence of overt toxicity related to the adenovirus or to transgene expression. Light microscopy revealed no evidence of pathologic changes in the organs of the animals treated with the adenoviral vectors compared with PBS treatment.

The organs from the rats treated with Ad.lacZ and PBS were processed with X-gal chromagen to identify uptake of the adenoviral transgene by hematogenous dispersion. Skeletal muscle and thoracic aorta were negative for β-galactosidase. Rarely did a cell or cluster of cells (one or two cells per sagittal section of liver lobe) show evidence of blue staining in the liver.

DISCUSSION

bFGF has been shown to be synthesized in the neointima, but it has not been established that ongoing synthesis is required for the response to arterial injury. We hypothesized that expression of an ASbFGF RNA, initiated through adenoviral gene transfer at the time of injury, would block new synthesis of bFGF during the critical time when SMCs undergo proliferation and migration. Antisense inhibition of bFGF gene expression should have no effect on preformed bFGF stores synthesized before arterial in-

jury, thus providing a means of distinguishing between the roles of these two sources of bFGF. By measuring the I/M ratio 2 weeks after injury, the influence of blocking bFGF synthesis after gene transfer was assessed.

A statistically significant decrease in intimal thickening resulted from the moderate and high doses of Ad.ASbFGF, and the high dose of Ad.ASbFGF almost completely inhibited intimal thickening. To attribute specificity of the results to the Ad.ASbFGF transgene rather than to toxicity from the vector, we used the same adenoviral vector system to deliver the Ad.lacZ reporter gene. All such adenovirally treated controls demonstrated neointimal thickening, as did the PBS treated controls, without significant difference noted between the mean I/M ratios of these control groups.

These results demonstrate that Ad.ASbFGF inhibits intimal thickening at 2 weeks in a dose-dependent fashion. These results differ from the results of Olsen et al.[8] results using neutralizing antibody to bFGF. In their study, neutralizing antibody given once at the time of injury had no effect on intimal thickening at 8 days but did reduce the SMC proliferative index at 41 hours. These results may be a result of rapid clearance of the neutralizing antibody soon after systemic delivery so that it was not present during the critical time of SMC migration and proliferation. Alternatively, because the neutralizing antibody was delivered systemically, it may not have had access to biologically important cytosolic or nuclear sites of bFGF activity.

This study demonstrates that an adenovirally expressed antisense bFGF RNA, delivered to the arterial wall at the time of arterial balloon injury, is a potent inhibitor of subsequent intimal thickening. These in vivo results imply that the arterial reparative response relies directly or indirectly on the de novo synthesis of bFGF after arterial injury, although the mechanism remains to be proven. Adenovirally mediated gene transfer of ASbFGF RNA may represent a novel local therapy to enhance the efficacy of peripheral and coronary interventions.

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